Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5903–5907, December 1978
Biochemistry

Altered nucleosome spacing in newly replicated chromatin from Friend leukemia cells

(nucleosomes/chromatin replication/staphylococcal nuclease)

ROBERT F. MURPHY, R. BRUCE WALLACE*, AND JAMES BONNER

Division of Biology, California Institute of Technology, Pasadena, California 91125

Contributed by James Bonner, September 18, 1978

ABSTRACT **Chromatin from Friend leukemia cells labeled with [^{14}C]thymidine for 24 hr followed by [^{3}H]thymidine for 10 min is converted into nucleosomes by staphylococcal nuclease at only half the rate that total chromatin is converted. Polyacrylamide gel electrophoresis of nucleosomal DNA from cells labeled for 24 hr with [^{14}C]thymidine followed by 10 min with [^{3}H]thymidine demonstrates that the internucleosomal spacer of newly replicated chromatin is approximately 20 base pairs shorter than that of total chromatin. The implications of this difference for models of chromatin structure are discussed.**

Considerable evidence from a number of laboratories indicates that the bulk of eukaryotic DNA exists in a repeated, globular form [reviewed by Kornberg (1)]. The repeat unit (the nucleosome), contains 140 base pairs of DNA associated with an octamer of the "inner" histones (H2A, H2B, H3, and H4), and between 40 and 80 base pairs in an internucleosomal spacer. Recent experiments suggest that 30–50 base pairs of this spacer are complexed with histone H1 (2–4). Variations in the spacer length have been observed between species and cell types (5–11), and the breadth of the bands observed in DNA gels has led to the postulation of heterogeneity within a single cell type (12). Investigations have so far revealed no differences in nucleosome spacing between transcribed and untranscribed chromatin (13–18). In this report we present evidence of a difference in repeat length between newly replicated and total chromatin from Friend leukemia cells.

MATERIALS AND METHODS

Cell Culture and Labeling. Friend leukemia cells, clone FSD-3, were grown in suspension culture as described previously (19). Cells were labeled with [^{14}C]thymidine (61 Ci/mol, Moravek Biochemicals) at 0.1 μCi/ml for 24 hr (1.5 generations) followed by [^{3}H]thymidine (21 Ci/mmol, Amersham) at 10 μCi/ml for 10 min or at 4 μCi/ml for 30 min (1 Ci = 3.7×10^{10} becquerels).

Preparation of Nuclei and Nucleosomes. Cells were harvested at the end of the ^{3}H labeling period, washed once in phosphate-buffered saline, and suspended in 10 mM Tris·HCl, pH 8.0/10 mM NaCl/5 mM $Mg(OAc)_2$/0.5% Nonidet-P40 (Shell Chemical). After 10 min, a nuclear pellet was formed by centrifugation at 2000 × *g* for 2 min. The nuclei were washed once in Tris/NaCl/Mg/Nonidet-P-40 and once in Tris/NaCl/Mg and were resuspended in Tris/NaCl/Mg/0.25 mM $CaCl_2$. The suspension was adjusted to approximately 50 μg of DNA per ml and brought to 37°C. Staphylococcal nuclease (P-L Biochemicals) was added to aliquots of the nuclear suspension at concentrations of 0.01–65 units/ml (1 unit produces 15 A_{260} units of acid-soluble material from DNA in 30 min at pH 8.8, 37°C), and digestion was stopped after 5 min by addition of 4 vol of ice-cold Tris/NaCl/Mg. The reaction mix was centrifuged at 2000 × *g* for 2 min, and the soluble material was removed (this fraction was found to be equivalent to the fraction soluble in 5% perchloric acid). The pellet was suspended in 2.5 mM Tris·HCl, pH 8.0/2.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). The Tris/EGTA-insoluble (undigested) fraction was removed by centrifugation at 2000 × *g* for 3 min. The amount of DNA in the various fractions was determined by bringing aliquots to 1% sodium dodecyl sulfate and scintillation counting in Aquasol-2 (New England Nuclear). A ^{14}C to ^{3}H spilldown correction of 17% was performed (the labeling conditions produced roughly equal amounts of ^{3}H and ^{14}C cpm). Under these counting conditions, the sum of the cpm in the three fractions for each isotope was constant from low to high enzyme concentration, and equal to the total input cpm. Thus the differences observed were not due to preferential quenching of large [^{3}H]DNA. In addition, no differences between 24-hr ^{14}C-labeled and 24-hr ^{3}H-labeled DNA were seen (data not shown).

Preparation of DNA and Gel Electrophoresis. DNA was extracted from digested nuclei essentially by the procedure of Marmur (20) and dissolved in 89 mM Tris/89 mM boric acid/2.5 mM EDTA containing 5% (vol/vol) glycerol and 0.01% bromophenol blue. Approximately 10 μg of the DNA samples was applied to 15-cm 4% polyacrylamide gels [in Tris/borate/EDTA, (21)] and electrophoresed at 150 V for 2 hr. Gels were fractionated into 2-mm slices (Aliquogel fractionator, Gilson) and scintillation counted in Aquasol-2. Data were corrected for spilldown of ^{14}C and converted to mobility relative to the dye. The replicative form 2 DNA of phage φX174 was cleaved with restriction endonuclease *Hae* III and labeled by a slight modification of the procedure of Berkner and Folk (22).

RESULTS

We have examined the digestion by staphylococcal nuclease of DNA in isolated nuclei from cells that had been labeled for 24 hr with [^{14}C]thymidine followed by 10 or 30 min with [^{3}H]thymidine. The amount of radioactivity in acid-soluble, Tris/EGTA-soluble and Tris/EGTA-insoluble form was determined at various ratios of enzyme to substrate. [From analysis of sucrose gradient profiles, the Tris/EGTA-soluble fraction represents nucleosome multimers from 1 to about 20 (data not shown).] Fig. 1 and Table 1 show that 30-min-labeled chromatin is digested into nucleosomes at about ⅔ the rate, and 10-min-labeled chromatin at less than ½ the rate that total chromatin is digested. However, the fact that there is no difference in the production of acid-soluble material suggests a difference in the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

* Present address: Department of Biology, City of Hope National Medical Center, Duarte, CA 91010.

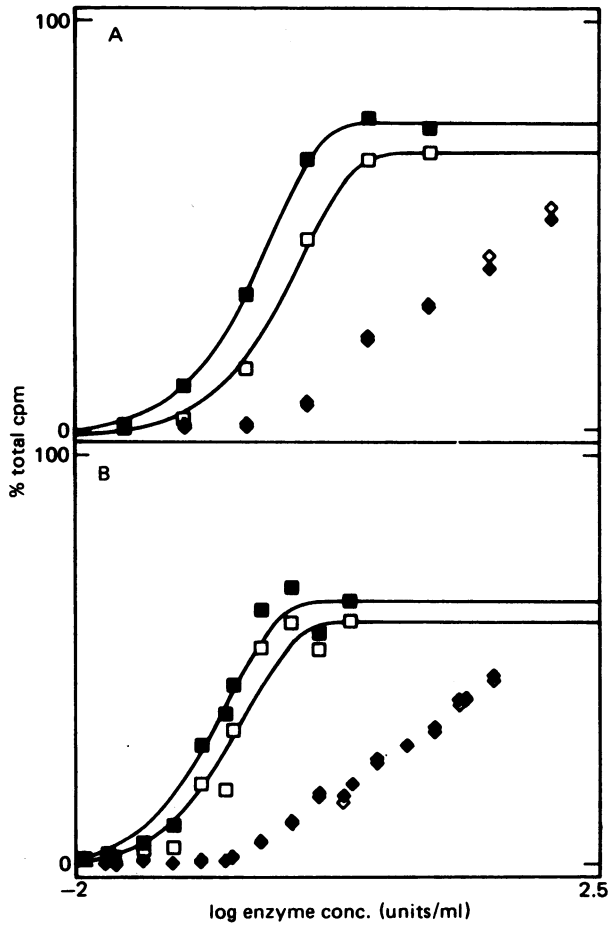

FIG. 1. Digestion of newly labeled and total chromatin by staphylococcal nuclease. The percent of total cpm in each fraction (P) is plotted versus the log of enzyme concentration (E, units/ml). (This semilogarithmic plot expands the low enzyme region.) (A) Ten-minute labeling, ^3H total cpm = 35,421 (±6.3%), ^{14}C total cpm = 13,710 (±8.3%) (uncertainties are % SD). (B) Thirty-minute labeling, data from three experiments. ^3H total cpm = 164,524, 110,084, 167,690; ^{14}C total cpm = 46,223, 100,724, 100,750 (all ±3–4%). ♦, ^{14}C (total) Tris/NaCl/Mg-soluble (acid-soluble); ◊, ^3H (new) Tris/NaCl/Mg-soluble (acid-soluble); ■, ^{14}C (total) Tris/EGTA-soluble (nucleosomes); □, ^3H (new) Tris/EGTA-soluble (nucleosomes). The lines represent fits to the equation $P = Me^{kE}$ drawn by using a nonlinear least-squares fitting program (R. F. Murphy, W. R. Pearson, and J. Bonner, unpublished and ref. 23) (see Table 1).

initial availability of internucleosomal DNA. The accuracy of these estimates depends in large part on the adherence of nuclease digestion to pseudo-first-order kinetics under our conditions. This problem may be avoided by plotting the same data as $^{14}C/^3H$ ratio (Fig. 2). It can be seen that the difference is

Table 1. Kinetic parameters from Fig. 1

Chromatin	k*	M†	R‡
10-min new	0.224	70.2	15.7
10-min total	0.416	76.9	32.0
Ratio new/total	0.539	0.912	0.491
30-min new	0.665	61.4	40.9
30-min total	0.956	66.8	63.9
Ratio new/total	0.696	0.920	0.640

* First-order rate constant (ml unit^{-1} min^{-1}).
† Maximum percent digested.
‡ Initial rate of reaction ($k \times M$).

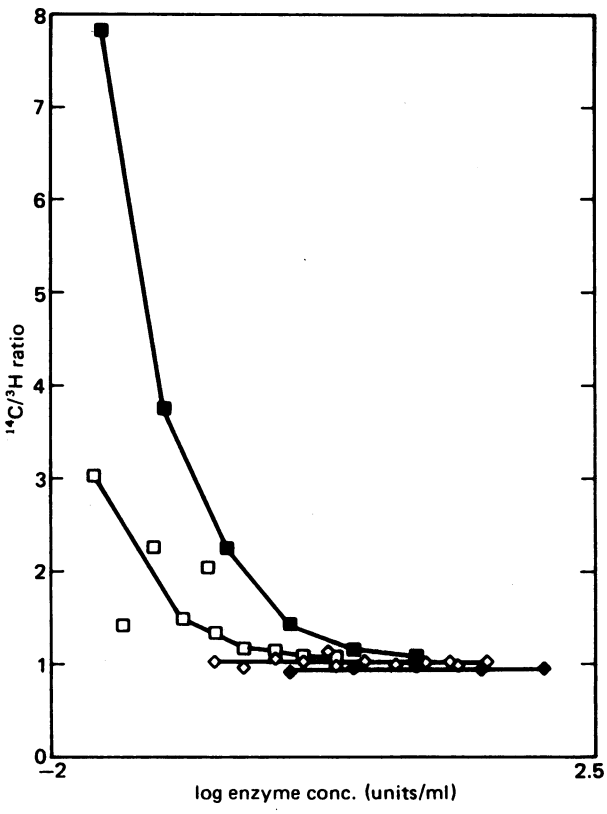

FIG. 2. Comparison of digestion of total and newly labeled chromatin. Data from Fig. 1 replotted as $^{14}C/^3H$ ratio for each sample. ♦, 10-min Tris/NaCl/Mg-soluble; ◊, 30-min Tris/NaCl/Mg-soluble; ■, 10-min Tris/EGTA-soluble; □, 30-min Tris/EGTA-soluble.

greatest at low enzyme-to-chromatin ratios, approaching 8-fold for a 10-min labeling.

The difference in digestion rates observed suggests a difference in the structure of newly replicated chromatin. In order to examine that possibility, total DNA from various digests of nuclei labeled for 24 hr with [^{14}C]thymidine followed by 10 min with [^3H]thymidine was prepared and electrophoresed in 4% polyacrylamide gels (Fig. 3). The monomer peaks in each gel are coincident, but a difference in the higher multimers is evident. The ^3H peaks appear broader, so that for dimers and trimers the ^{14}C peak is almost included in the ^3H peak. The panels on the right show the data plotted as $^3H/^{14}C$ ratio. Peaks are seen to the right of the position of the ^{14}C-labeled multimers (marked by the arrows), demonstrating a difference in the average size of the two types of multimer DNA.

To quantitate this difference, Gaussian curves were fit to the data from each gel for each isotope by using a nonlinear least-squares fitting program (R. F. Murphy, W. R. Pearson, and J. Bonner, unpublished; ref. 24). ^{32}P-Labeled fragments of ϕX174 replicative form 2 DNA cleaved with *Hae* III were either run on parallel gels or mixed with the ^3H and ^{14}C samples. These gels were also fractionated and their radioactivities were determined. The mobilities of these fragments were 1–2% higher at low DNA concentrations (approximately 100 ng/gel), compared to the same fragments mixed with the Friend cell DNA samples (approximately 10 μg/gel; data not shown). For the standardization, values from the latter gels were used. Fig. 4 shows the nucleotide length of each fragment [determined from the nucleotide sequence (25)] plotted semilogarithmically versus its mobility. This yields a straight line, from which the sizes of the ^3H and ^{14}C nucleosomes were calculated.

Fig. 5 shows the variation in nucleosome size with multimer number, from which the nucleosome repeat length can be de-

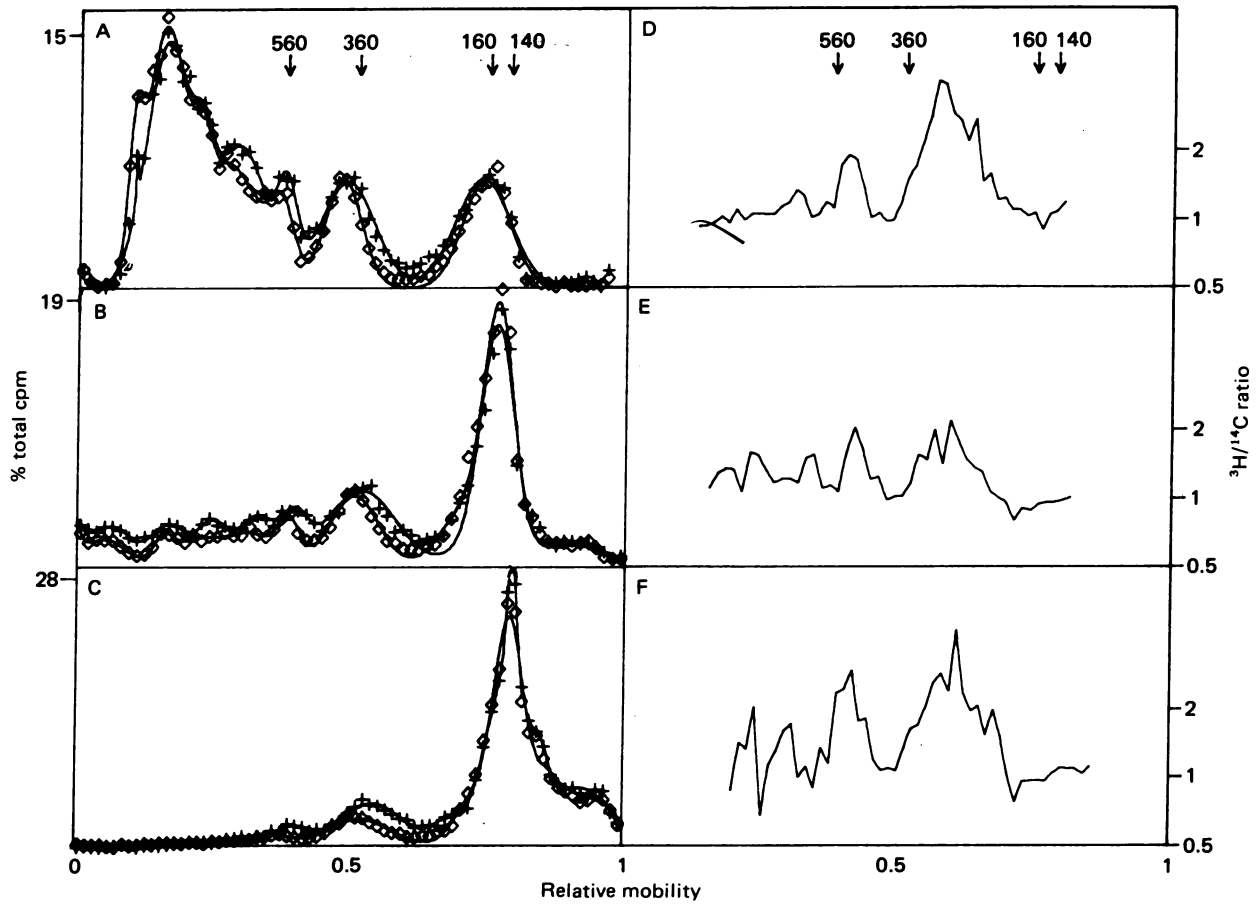

FIG. 3. Polyacrylamide (4%) gel electrophoresis of DNA from newly labeled and total nucleosomes. (*A–C*) Percent of total nuclear cpm for ^3H (+, new) and ^{14}C (◇, total) nucleosomal DNA is plotted versus mobility relative to bromophenol blue. ^3H total cpm: *A*, 4019; *B*, 4571; *C*, 3605. ^{14}C total cpm: *A*, 5772; *B*, 6259; *C*, 4873. The lines represent the sum of Gaussian curves fit to the data by using a nonlinear least-squares fitting program (R. F. Murphy, W. R. Pearson, and J. Bonner, unpublished). The arrows mark the approximate position of the ^{14}C core and multimers, and the associated numbers of base pairs were calculated from the standards as described in the text. The enzyme concentration increases 3.16-fold from *A* to *B* and from *B* to *C*. (*D–F*) Ratio of $^3H/^{14}C$ cpm from *A–C*.

termined independently of the extent of digestion. Table 2 summarizes this data. The difference in size is clearly due to a difference in spacer length, which varies from 10 base pairs in the least digested sample to 29 in the most. Because the breadth of the bands appears to decrease with digestion, the average value of 17 is probably an underestimate.

Table 2. Nucleosomal DNA sizes

	Chromatin	Mono	Di	Tri	Unit	Spacer*	Spacer difference
A†	New	168	387	563	198	58	
	Total	164	397	579	208	68	10
B†	New	151	349	528	188	48	
	Total	150	365	548	199	59	11
‡	New	144	331		187	47	
	Total	148	358		210	70	23
C†	New	140	323	527	194	54	
	Total	139	348	585	223	83	29
‡	New	138	320		182	42	
	Total	140	336		195	55	13
	Average						17 ± 8

* Assuming a core size of 140 base pairs.
† Fig. 3 panel number.
‡ Gel not shown; enzyme concentration as for preceding gel.

DISCUSSION

Much evidence has been accumulated to indicate that nucleosomes from different species and cell types have different repeat lengths (5–11). A correlation between nucleosome size and transcriptional activity has been suggested (9–10). As has been pointed out by Thomas and Thompson (11), however, it would appear unlikely that two different cells from the same organism would have a significant difference in their average repeat size because of differences in the small fraction of total DNA coding for structural genes. Some reports of the presence of transcribed genes in nucleosome structures have included data that indicate that the spacing for transcribed and nontranscribed DNA is the same. Two of these methods—comparison of unlabeled DNA in parallel gels (17) and comparison of unlabeled total DNA and labeled, hybridized probe for the examined sequence (15, 16, 18)—are severely limited in their accuracy due to difficulties in aligning and scaling the different sets of data. The third method—double-labeling—has been used to show that 60- to 120-min labeled ribosomal DNA and long-term labeled total DNA from *Tetrahymena* macronuclei nucleosomes have the same size (13, 14). No direct evidence of a relationship between nucleosome spacing and transcriptional activity has been reported.

As we have shown above, newly replicated nucleosomal DNA from mouse Friend cells is different in size from total nucleo-

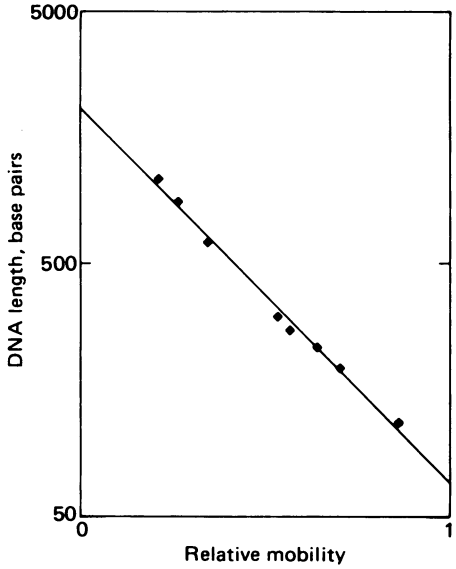

FIG. 4. Molecular weight standardization curve for 4% polyacrylamide gels. DNA length L (base pairs) is plotted semilogarithmically versus mobility relative to bromophenol blue for ϕX174 replicative form DNA cut with *Hae* III [♦, lengths determined from the DNA sequence (24)]. The line represents the least-squares fit log $L = 3.308 - 1.473\,R_F$ (correlation coefficient $r = -0.9963$, % error = 1.06) for the ϕX174 markers, whose lengths are 118, 194, 234, 271 and 278, 310, 606, 872, and 1078 base pairs.

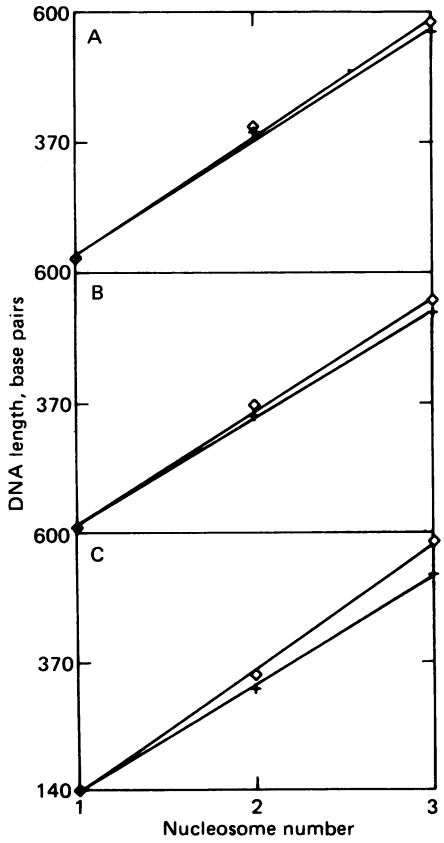

FIG. 5. Determination of nucleosome spacing. DNA length (base pairs, determined from the center of fitted Gaussian curves) is plotted versus nucleosome multimer number (n) for the gels from Fig. 3. +, ^{3}H; ◇, ^{14}C. The lines are least-squares fits $L = a + bn$, in which a is the sum of the lengths of the ends on each multimer minus the spacer length and b is the unit nucleosome size (core length plus the spacer length) (see Table 2).

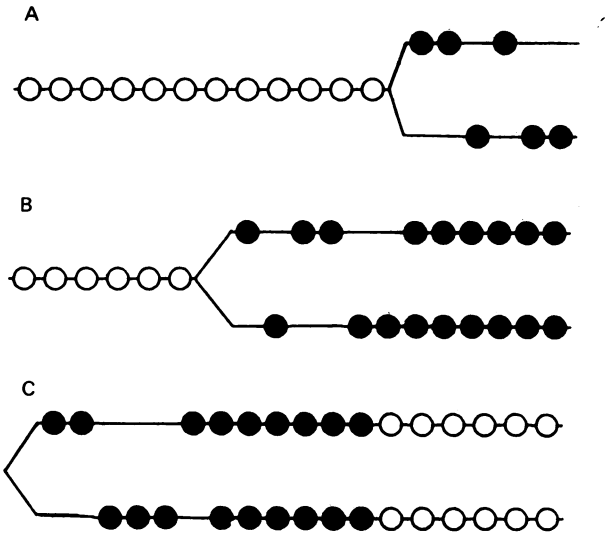

FIG. 6. Changes in nucleosome arrangement during chromatin replication. All circles represent 140-base-pair nucleosome cores. ○, nucleosomes with normal (200-base-pair) spacing; ●, nucleosomes with shortened (180-base-pair) spacing. The number of nucleosomes depicted in each configuration is arbitrary. In reality, the number of nucleosomes (and hence the length of time) between the passage of the replication fork and regaining of normal spacing is certainly much larger. (*A*) Chromatin after replication has been initiated. Compression of nucleosomes has occurred to allow for replication complex. A random dispersive model is shown, but semiconservative and conservative models are not ruled out. Current data are conflicting in this regard, but the proposed compression during replication is independent of the exact dispersion mechanism. Nuclease digestion of chromatin labeled for short times (1–30 sec, depending on the rate of replication for the specific cell type) yields an increased rate of production of both acid-soluble material and mononucleosomes. (*B*) New nucleosome cores cover the free DNA, but shortened spacing remains intact. Additional free DNA generated by compression is present at the fork. Longer pulses (5–10 min) detect no difference in production of acid-soluble material, decreased oligonucleosome production, and shortened nucleosome spacing. (*C*) Normal spacing is slowly regained by nucleosome sliding (20–30 min). The amount of free DNA at the fork is thus kept constant.

somal DNA. This might suggest a relationship between nucleosome spacing and chromatin replication rate, because the general correlation between shortened nucleosomal spacings and high transcriptional activity [reviewed by Kornberg (1)] can also be made between shortened spacing and short generation times. However, there is no difference in spacing between Chinese hamster ovary cell nuclei and mitotic chromosomes (17), or between confluent and exponentially growing C6 rat glial tumor cells (10). These experiments used unlabeled DNA in parallel gels, and hence do not have the sensitivity of our experiments. However, the conclusion that nucleosome spacing is not significantly related to rate of chromatin replication is probably justified, because the fraction of chromatin in newly replicated form is too small to account for spacing differences in total DNA from cells with different generation times. Instead, a gradual increase in nucleosome spacing with time is possible (perhaps to compensate for small amounts of histone degradation or as a result of changes in the level of histone modification).

Some laboratories have reported that chromatin pulse-labeled for from 0.5 to 10 min produces acid-soluble material at an increased rate (relative to total chromatin) when digested with staphylococcal nuclease (26–28). These studies have generally used shorter pulses than we have, and those that have used longer pulses have indicated that the difference disappears (26,

28). Considering the variation in chromatin replication rate among different cell lines, it would appear that the published data are consistent with an increased sensitivity during and immediately following DNA replication and normal sensitivity once histone cores are deposited on the free DNA. The short-lived acid-soluble difference is followed by a longer-term decrease in production of nucleosomes by staphylococcal nuclease, due to smaller nucleosome spacers (Fig. 6).

In any case, our results indicate that elongation can take place after histone deposition (perhaps caused by the binding of histone H1 and/or some nonhistone protein). This suggests that the interaction between core histones and DNA is not strong enough to prevent nucleosome "sliding," a conclusion that may be significant for models of chromatin replication and transcription.

After this manuscript was prepared, Levy and Jakob (29) and Seale (30) reported similar results for sea urchin embryos labeled for 7 sec and HeLa cell nuclei labeled *in vitro* for 20 min, respectively.

ϕX174 DNA cleaved with *Hae* III was the gift of Richard Parker. We thank Thomas Sargent for labeling the ϕX174 DNA and for helpful discussion of this manuscript. This work was supported by U.S. Public Health Service Grant GM-13762. R.F.M. is a National Institutes of Health predoctoral trainee. R.B.W. is the holder of a Medical Research Council of Canada Centennial fellowship.

1. Kornberg, R. D. (1977) *Annu. Rev. Biochem.* **46,** 931–954.
2. Whitlock, J. P., Jr. & Simpson, R. T. (1976) *Biochemistry* **15,** 3307–3314.
3. Bakayev, V. V., Bakayeva, T. G. & Varshavsky, A. J. (1977) *Cell* **11,** 619–629.
4. Noll, M. & Kornberg, R. D. (1977) *J. Mol. Biol.* **109,** 393–404.
5. Noll, M. (1976) *Cell* **8,** 349–355.
6. Morris, N. R. (1976) *Cell* **8,** 357–363.
7. Thomas, J. O. & Furber, V. (1976) *FEBS Lett.* **66,** 274–280.
8. Spadafora, C., Bellard, M., Compton, J. L. & Chambon, P. (1976) *FEBS Lett.* **69,** 281–285.
9. Morris, N. R. (1976) *Cell* **9,** 627–632.
10. Compton, J. L., Bellard, M. & Chambon, P. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4382–4386.
11. Thomas, J. O. & Thompson, R. J. (1977) *Cell* **10,** 633–640.
12. Lohr, D., Corden, J., Tatchell, K., Kovacic, R. T. & van Holde, K. E. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 79–83.
13. Piper, P. W., Celis, J., Kaltoft, K., Lerr, J. C., Nielsen, O. F. & Westergaard, O. (1976) *Nucleic Acids Res.* **3,** 493–505.
14. Mathis, D. J. & Gorovsky, M. A. (1976) *Biochemistry* **15,** 750–755.
15. Bokhon'ko, A. & Reeder, R. H. (1976) *Biochem. Biophys. Res. Commun.* **70,** 146–152.
16. Bostock, C. J., Christie, S. & Hatch, F. T. (1976) *Nature (London)* **262,** 516–519.
17. Compton, J. L., Hancock, R., Oudet, P. & Chambon, P. (1976) *Eur. J. Biochem.* **70,** 555–568.
18. Gottesfeld, J. M. & Melton, D. A. (1978) *Nature (London)* **273,** 317–319.
19. Dube, S. K., Gaedicke, G., Kluge, N., Weimann, B. J., Melderis, H., Steinheider, G., Crozier, T. & Ostertag, W. (1974) in *Proceedings of the 4th Symposium: Princess Takamatsu Cancer Research Fund, Differentiation and Control of Malignancy in Tumor Cells* (Univ. of Tokyo Press, Tokyo), pp. 99–136.
20. Marmur, J. (1961) *J. Mol. Biol.* **3,** 208–218.
21. Maniatis, T., Jeffrey, A. & van deSande, H. (1975) *Biochemistry* **14,** 3787–3794.
22. Berkner, K. L. & Folk, W. R. (1977) *J. Biol. Chem.* **252,** 3176–3184.
23. Pearson, W. R., Davidson, E. H. & Britten, R. J. (1977) *Nucleic Acids Res.* **4,** 1727–1737.
24. Wallace, R. B., Sargent, T. D., Murphy, R. F. & Bonner, J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3244–3248.
25. Sanger, F., Air, G. M., Barrel, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M. & Smith, M. (1977) *Nature (London)* **265,** 687–695.
26. Seale, R. L. (1975) *Nature (London)* **255,** 247–249.
27. Hildebrand, C. E. & Walters, R. A. (1976) *Biochem. Biophys. Res. Commun.* **73,** 157–163.
28. Burgoyne, L. A., Mobbs, J. D. & Marshall, A. J. (1976) *Nucleic Acids Res.* **3,** 3293–3304.
29. Levy, A. & Jakob, K. M. (1978) *Cell* **14,** 259–267.
30. Seale, R. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2717–2721.